

ORIGINAL ARTICLE

Quantitative Identification of Compound-Dependent On-Modules and Differential Allosteric Modules From Homologous Ischemic Networks

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Module-based methods have made much progress in deconstructing biological networks. However, it is a great challenge to quantitatively compare the topological structural variations of modules (allosteric modules [AMs]) under different situations. A total of 23, 42, and 15 coexpression modules were identified in baicalin (BA), jasminoidin (JA), and ursodeoxycholic acid (UA) in a global anti-ischemic mice network, respectively. Then, we integrated the methods of module-based consensus ratio (MCR) and modified Z_{summary} module statistic to validate 12 BA, 22 JA, and 8 UA on-modules based on comparing with vehicle. The MCRs for pairwise comparisons were 1.55% (BA vs. JA), 1.45% (BA vs. UA), and 1.27% (JA vs. UA), respectively. Five conserved allosteric modules (CAMs) and 17 unique allosteric modules (UAMs) were identified among these groups. In conclusion, module-centric analysis may provide us a unique approach to understand multiple pharmacological mechanisms associated with differential phenotypes in the era of modular pharmacology.

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Study Highlights

WHAT IS THE CURRENT KNOWLEDGE ON THE TOPIC?

Module-based methods, rather than independent genes or proteins, have made much progress in deconstructing the complex networks and were prospected in contributing the rational drug design paradigm.

WHAT QUESTION DID THIS STUDY ADDRESS?

There is a great challenge to quantitatively compare topological structural variations of modules in different situations. We used an integrated method of module-based consensus ratio and modified Z_{summary} statistics to validate compound-dependent on-modules based on comparing the pharmacologic actions.

WHAT THIS STUDY ADDS TO OUR KNOWLEDGE

Conserved AMs of BA, JA, and UA revealed their common mechanisms in anticerebral ischemia, such as the MAPK and calcium-signaling pathway, and unique AMs found their divergent biological functions, such as the BA Hedgehog signaling pathway.

HOW THIS MIGHT CHANGE DRUG DISCOVERY, DEVELOPMENT, AND/OR THERAPEUTICS

The AM identification may help to explore the therapeutic target modules rather than a single gene or protein in disease therapy. In addition, this module-centric analysis may provide a unique path to reveal multiple pharmacologic mechanisms associated with differential phenotypes.

There is increasing evidence that both pathogenesis of diseases and mechanism of action of drugs have a module basis, as genes and proteins should interact with each other in the network to execute certain functions.^{1–3} Module-based methods have made much progress in deconstructing complex networks and may contribute substantially to rational drug design in the context of modular pharmacology.^{4,5} Several studies have attempted to identify module biomarkers or targets for cancers and many other diseases.^{6–11} Causal coexpression methods with module analysis have been applied to screen drugs with specific targets and fewer side effects.¹² Such module-targeting approaches rather than targeting at independent genes or proteins may provide us an intensive understanding of the underlying mechanisms of drug actions.¹³ In addition, actions of functionally similar drugs in treating the same disease can be

comparatively analyzed based on modular functions. However, when different drugs are used to affect the same disease network, their common and specific modular relationships are often neglected.

In the gene coexpression network, the correlation patterns among genes across microarray samples are described as the gene relationship significance, the significant gene relationships have a coexpression edge. These highly correlated genes often coordinate together as a functional cluster, so the densely interconnected clusters are defined as coexpression modules. Under different conditions, such as the treatment with different drugs, the gene coexpression relationship can be changed and manifest as an intramodular edge rewiring, which reflects the different condition responses, so we defined the significant topological structure-changed modules under different conditions

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as allosteric modules (AMs). In this study, coexpression and AM-based analysis was applied to elucidate and compare the pharmacological mechanisms of three drugs in treating cerebral ischemia.

Baicalin (BA), jasminoidin (JA), and ursodeoxycholic acid (UA) are bioactive ingredients extracted from Qingkailing, a traditional Chinese medicine formula that is effective and widely used in treating patients who undergo a stroke in China.¹⁴ The pharmacological actions of BA mainly include neuroprotection, anti-inflammation, and antioxidation,^{15–17} and BA may act on TLR2/4 signaling pathway, antioxidative, and antiapoptotic pathways, GABAergic signaling, HSP70, and mitogen-activated protein kinase (MAPK) cascades, as well as the PI3K-Akt-PKB-BAD-CREB-PCREB pathway.^{14,16–18} The pharmacologic activities of JA include neuroprotection, choleric action, enzyme inhibition, and anti-inflammation,¹⁹ and it may act on the NF- γ B pathway, PI3K pathway, TLR4 pathway, and MAPK pathway.^{20–23} As for UA, it plays a unique role in modulating the apoptotic threshold in both hepatic and nonhepatic cells, and it may inhibit apoptosis by either stabilizing the mitochondrial membrane or modulating the expression of specific upstream targets.^{24,25} It has been shown that BA, JA, and UA all exert effects on multiple pathways in animal models of cerebral ischemia.²⁶ Previous studies compared the functions of these three drugs based on differentially expressed genes as well as protein-protein interaction networks, but the convergent or divergent modules of different drugs were not identified.²⁷

In this study, we used the method of weighted gene coexpression network analysis (WGCNA)²⁸ to identify coexpression modules of BA, JA, and UA in a global anti-ischemic mice network consisting of 374 stroke-related cDNAs. The module-based consensus ratio (MCR) and Z_{summary} module preservation statistic were used to validate compound-dependent on-modules, and the conserved allosteric modules (CAMs) and unique allosteric modules (UAMs) were also identified. Then, modular analysis based on the drug-induced gene coexpression and functional changes was performed to illuminate and compare the underlying pharmacological mechanisms of BA, JA, and UA in treating cerebral ischemia.

MATERIALS AND METHODS

Gene expression datasets

Expression data, which originated from our previous studies,²⁹ were obtained from ArrayExpress database (<http://www.ebi.ac.uk/arrayexpress/>, E-TABM-662; see **Supplementary Text S1**). Microarrays were constructed from a collection of 374 cDNAs related to cerebral ischemia (Clontech Atlas 1.2 mouse brain microarray, “Biostar40S” 4065, and 16,463 mouse oligo chips). The specific 374 genes and their expression level are listed in **Supplementary Table S1**. The procedures of RNA isolation, microarray preparation, and gene collections were described in refs. 19 and 30. Five groups of datasets were selected: sham group, vehicle group (VE) (0.9% NaCl), BA-treated group (5 mg/mL), JA-treated group (25 mg/mL), and UA-treated group (7 mg/mL).

Coexpression module identification

The construction and module identification of the gene coexpression networks were implemented following the protocols of the WGCNA R package.²⁸ A matrix of pairwise correlations was constructed between all pairs of probes across the measured samples by using appropriate soft-thresholding for each group ($\beta = 4$ for BA, 12 for JA, and 8 for UA), the thresholds were selected when the network gets the best scale-free topology criterion.³¹ To identify the coexpression modules, topologic overlap measure was used to perform average linkage hierarchic clustering, which got a dendrogram whose branches were identified using the Dynamic Hybrid Tree Cut algorithm,³¹ then, the branches were defined as modules and each module was subsequently assigned a color. We set the number three as the minimum module size in all three groups.

Module-based consensus ratio

We defined an MCR in Eq. 1 to compare the influence of different drugs on the gene coexpression level in the context of whole networks. The consensus module pairs were detected based on the overlapping genes within the two modules, and the Fisher's exact test was used to select significantly overlapped module pairs ($P < 0.05$). The MCR was defined as the ratio of significantly overlapped module pairs to all the module pairs between two drugs.

$$\text{MCR}_{a,b} = \frac{\text{NM}_{\text{overlap}}}{\text{NM}_a \times \text{NM}_b} \times 100\% \quad (1)$$

where NM represents the number of modules, a and b represent two different groups, and $\text{NM}_{\text{overlap}}$ represents the number of significantly overlapped module pairs between the two groups.

On-module and off-module

In order to quantitatively assess whether modules in the drug groups were changed in coexpression patterns independent of the vehicle, we adopted a Z_{summary} statistic implemented in the module preservation function of WGCNA, which can assess whether the density and connectivity patterns of modules defined in a reference dataset are preserved in a test dataset.³² A negative Z_{summary} value indicates the modules' disruption.³³ Compared to the vehicle, we defined a module with a negative Z_{summary} value as an on-module, which may be activated by a drug. On the other hand, a module with a positive Z_{summary} value is defined as an off-module. The equation of Z_{summary} is as follows³²:

$$Z_{\text{summary}} = \frac{\text{median}(Z_{\text{meanCor}}, Z_{\text{meanAdj}}, Z_{\text{propvarExpl}}, Z_{\text{meanKME}}) + \text{median}(Z_{\text{cor.KIM}}, Z_{\text{cor.KME}}, Z_{\text{cor.cor}})}{2}$$

Conserved allosteric module and unique allosteric module

The Z_{summary} value was also used to quantitatively assess whether a specific AM is conserved or unique compared with each drug group. A module with a Z_{summary} value > 2 indicates its preservation in a test dataset.³² So, compared

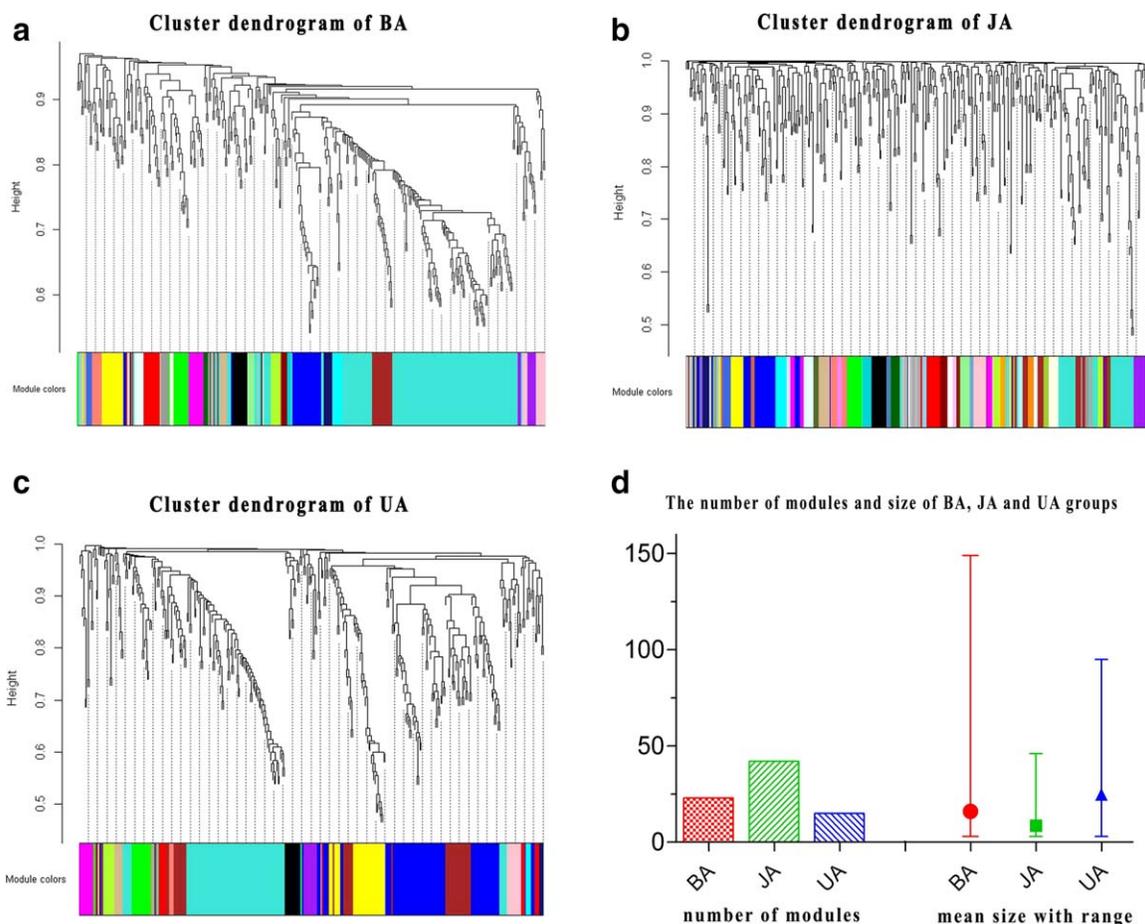


Figure 1 Hierarchic cluster tree and modules of the three drug groups. (a–c) The hierarchic cluster tree (dendrogram) of baicalin (BA), jasminoidin (JA), and ursodeoxycholic acid (UA), each major tree branch represents a module, and each module is labeled with a color below the dendrogram. (d) The number and size of the modules in the three groups are shown. The red, green, and blue bars represent the number of modules in the BA, JA, and UA groups, respectively. The red dot, green square, and blue triangle indicate the mean size of the modules in the BA, JA, and UA groups, respectively. The vertical line indicates the range of module size in each group.

to the test group, modules with a Z_{summary} value ≥ 2 are CAMs, which may be regarded as universal targets of two or more drugs. If a module has a negative Z_{summary} value compared with any other groups, this module is defined as a UAM, which reveals a specific target of this drug.

Functional annotation of modules

To characterize the function of modules, we performed Gene Ontology (GO) and KEGG pathway enrichment analysis using the Database for Annotation, Visualization, and Integrated Discovery.³⁴ For each module, a ranked list of the enriched functionally relevant annotation was provided. An overrepresentation of a term is defined as a modified Fisher's exact P value with an adjustment for multiple tests using the Benjamini method. In this analysis, all the genes on the array were set as the background, and GO terms and pathways with a $P < 0.05$ were considered as significant. To specify and simplify the enriched biological functions of modules, we classified the GO terms and pathways based on the GO slim³⁵ and KEGG functional hierarchies.

Western blotting analysis

The mitogen-activated protein kinase 6 (MAP2K6) protein was selected to validate the expression patterns in different groups. Standard Western blotting analyses were performed, as described previously.²⁶ The blots were probed with anti-MEK6 antibody (1:1000 dilution, ab71938; Abcam, UK), and β -actin (1:1000 dilution, Tdybio, TDY041, China) was used as an internal control. Western blot bands were quantified using QuantityOne software by measuring the band intensity (area \times outer diameter) for each group. The results are expressed as fold changes by normalizing the data to the control values.

RESULTS

Coexpression modules in the three drug groups

The gene coexpression network of BA, JA, and UA were constructed by WGCNA, as described in the Methods section. Hierarchic clustering procedures identified 23, 42, and 15 coexpression modules for BA, JA, and UA, respectively. Each module corresponded to a branch of the resulting

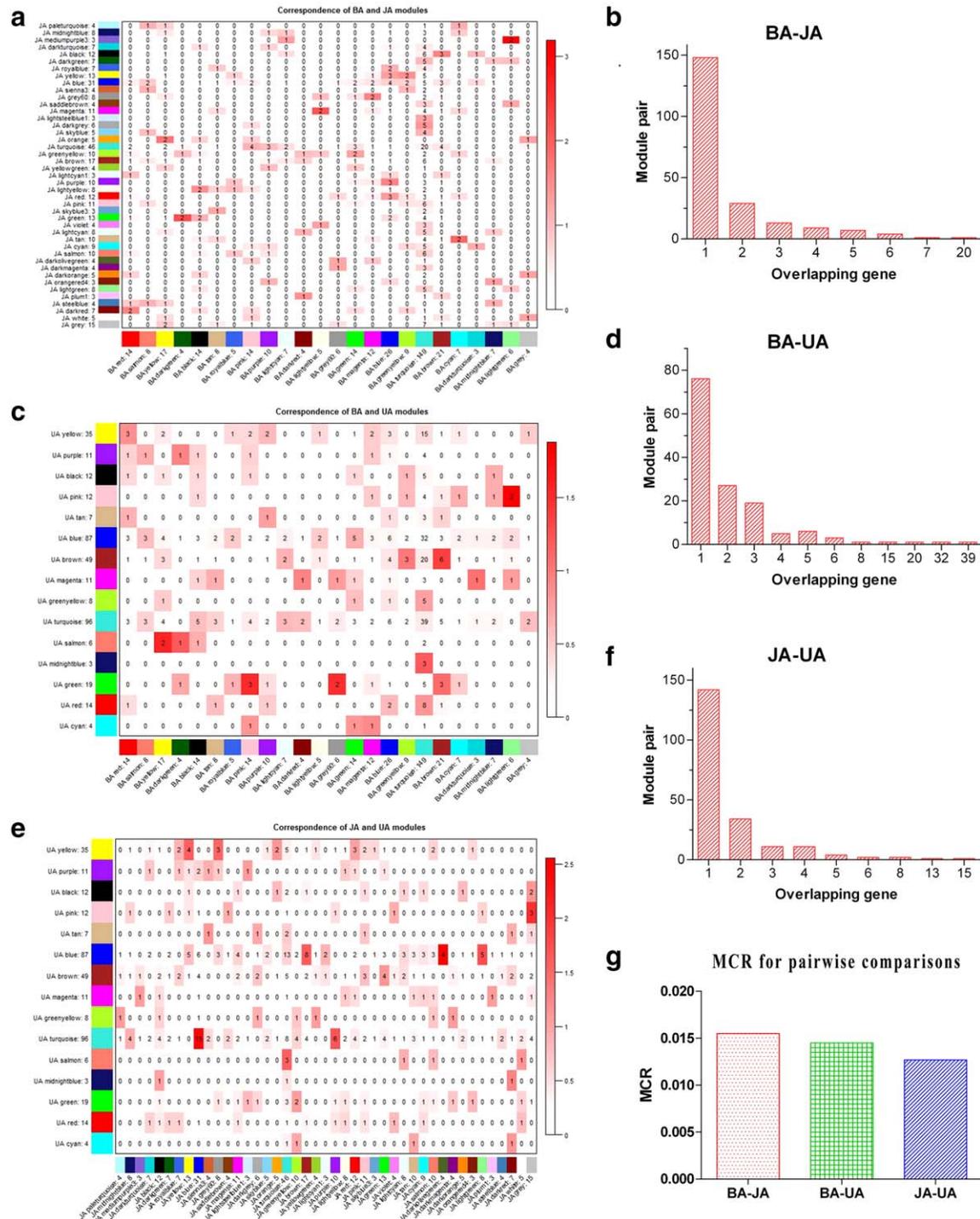


Figure 2 Concordance of modules among the three groups. (a) Concordance of modules between the baicalin (BA) and jasminoidin (JA) groups; each row of the table corresponds to the BA modules (labeled by color name and module size), and each column corresponds to the JA modules. Numbers in the table indicate gene counts in the intersection of the corresponding modules of the BA and JA groups. Coloring of the table encodes $-\log(P)$, with P being the Fisher's exact test P value for the overlap of the two modules. Any P value < 0.05 is considered significant. The darker the red color, the more significant the correlation. (b) The number of modules with a certain amount of overlapping genes between the BA and JA groups is shown. (c) Concordance of modules between the BA and ursodeoxycholic acid (UA) groups; the table legend is the same as panel a. (d) The number of modules with a certain amount of overlapping genes between the BA and UA groups. (e) Concordance of modules between the JA and UA groups; the table legend is the same as a. (f) The number of modules with a certain amount of overlapping genes between the JA and UA groups. (g) The module-based consensus ratios (MCRs) among the three drug groups.

clustering tree and labeled by a unique color (Figure 1a–c). The detailed modules of each group labeled by colors and numbers can be found in **Supplementary Table S1**. The average sizes (number of genes) of BA, JA, and UA modules were 16 (range, 3–149), 9 (range, 3–46), and 25 (range, 3–95), respectively (Figure 1d).

Concordance of modules among the three groups

To investigate the influence of the three drugs on gene coexpression levels in the context of whole networks, we compared the distribution of all genes in the modules of the three groups. Figure 2a–f shows the concordance of gene composition and the number of module pairs with a certain number of overlapping genes for each group's modules. The number of module pairs with at least five overlapping genes was small, and the MCRs for pairwise comparisons were only 1.55% (BA vs. JA), 1.45% (BA vs. UA), and 1.27% (JA vs. UA), respectively (Figure 2g), indicating a low level of in-module genes overlapping. Thus, there was a big difference in the gene coexpression level and module constitution among the three drug groups.

CAMs in each group

In order to quantitatively assess whether a specific AM of one drug group was conserved compared with the vehicle and other drug groups, we used a Z_{summary} statistic implemented in the module preservation function of WGCNA.³² A Z_{summary} value >2 indicates that the corresponding module is conserved. The CAMs and their Z_{summary} values with respect to different groups are listed in Table 1. Compared to the vehicle group, only the JA_2 and JA_18 modules were conserved. When pairwise comparisons were performed among the three drug groups, the JA_2 module was also conserved in the UA group, the JA_22 module was conserved in the BA group, the BA_5 module was conserved in both the JA and UA groups, the UA_2 module was conserved in the JA group, and the UA_11 module was conserved in the BA group. We named and visualized these CAMs in Figure 3.

Significant biological functions of CAMs

To characterize the biological function of the identified AMs, we performed GO term and KEGG pathway enrichment analysis. The most significant GO terms and pathways along with their P values of each CAM are listed in Table 1. All of the significant GO terms and pathways ($P < 0.05$) of the CAMs can be found in **Supplementary Table S2**. Among the top five significant functions (Figure 3), the BA_5, JA_2, and UA_2 from three groups were all enriched in MAPK signaling pathway; and both the BA_5 and JA_18 were enriched in neurotrophin signaling pathway. The UA_2 and JA_18 were both enriched in protein amino acid phosphorylation, phosphorylation GO terms, and pathways in cancer. Besides, amyotrophic lateral sclerosis was enriched by the JA_2 and UA_11, and GnRH signaling pathway was enriched by the JA_2 and UA_2.

On-modules and off-modules in the three groups

Our prior studies reported that BA, UA, and JA were effective in reducing the ischemic infarct volume compared to the vehicle group ($P < 0.05$).³⁶ Based on the detection and

Table 1 The Z_{summary} value and the most significant functions of CAMs
The CAMs across all groups

| Module names | No. of genes | Z_{summary} value for each test group | | | The most significant GO term | | The most significant KEGG pathway | | P value |
|--------------|--------------|--|-------|-------|------------------------------|---|-----------------------------------|-----------|-----------|
| | | VE | BA | JA | UA | Terms | Pathway | P value | |
| BA_5 | 14 | 0.40 | – | 2.30 | 2.30 | Calmodulin-dependent protein kinase activity | Calcium signaling pathway | 0.0198 | 0.0064 |
| JA_2 | 31 | 3.90 | –0.23 | – | 2.10 | Positive regulation of catalytic activity | MAPK signaling pathway | 8.89e-05 | 4.47e-07 |
| JA_18 | 8 | 2.60 | 0.69 | – | 0.02 | Protein amino acid phosphorylation | Pathways in cancer | 1.82e-04 | 0.0077 |
| JA_22 | 7 | 0.23 | 2.40 | – | –0.46 | NA | NA | – | – |
| UA_11 | 8 | 1.20 | 2.20 | –0.81 | – | Regulation of mitochondrial membrane permeability | ALS | 0.0041 | 0.0392 |
| UA_2 | 87 | –0.55 | 0.33 | 3.70 | – | Intracellular signaling cascade | MAPK signaling pathway | 2.21e-07 | 8.31e-11 |

Modules are named based on the name of the corresponding reference and test groups. For example, the green module in the BA group is conserved in the JA and UA groups, and we name it as CAM_BA (JA, UA). The following columns include data on the number of genes in the module, the Z_{summary} value for different test groups (bold numbers indicate the Z_{summary} value ≥ 2), the most significant GO terms and KEGG pathways with corresponding P values. ALS, amyotrophic lateral sclerosis; BA, baicalin; CAM, conserved allosteric module; GO, Gene Ontology; JA, jasminoidin; MAPK, mitogen-activated protein kinase; NA, not applicable; UA, ursodeoxycholic acid; VE, vehicle group.

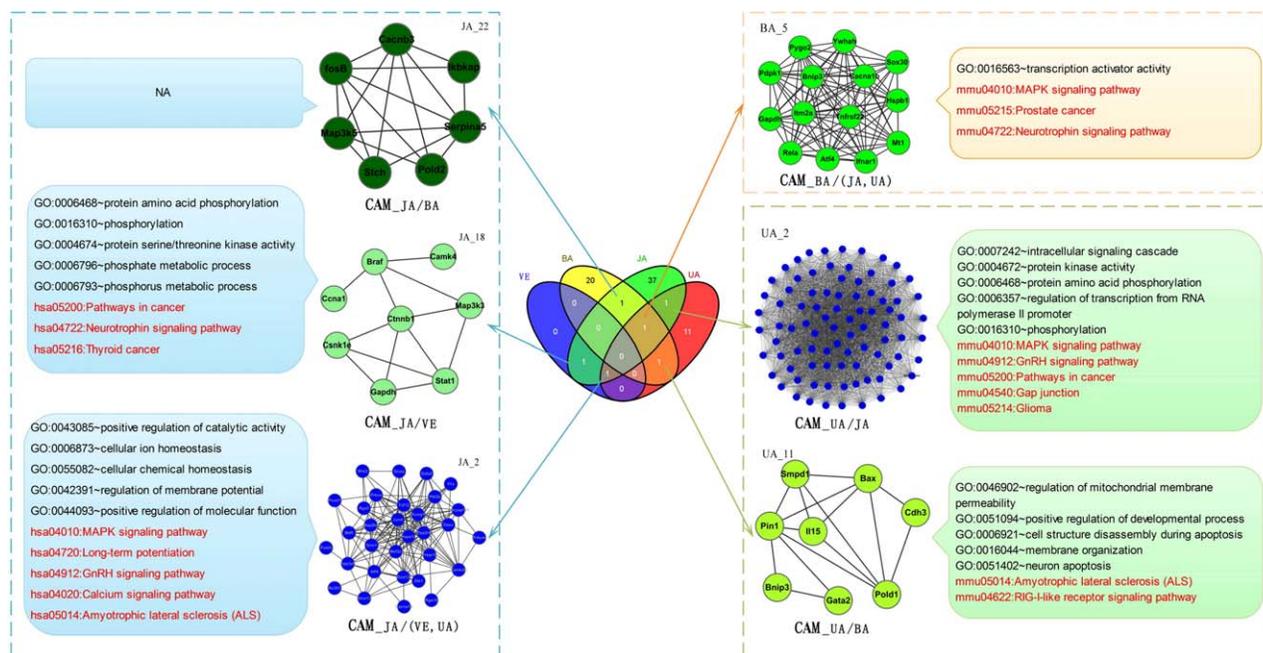


Figure 3 The conserved allosteric modules (CAMs) and their significant biological functions are shown. Modules in the orange dotted box are identified from the baicalin (BA) group, modules in the light blue dotted box are identified from the jasminoidin (JA) group, and modules in the light green dotted box are identified from the ursodeoxycholic acid (UA) group. The top five significantly enriched functions (black font color represents Gene Ontology (GO) terms and the red font color represents KEGG pathways) of each module are listed. The Venn diagram in the middle indicates to which conserved module belongs to which groups. MAPK, mitogen-activated protein kinase; NA, not applicable; VE, vehicle group.

statistical evaluation of changes in coexpression patterns, we also observed whether the modules in the three drug groups changed their gene coexpression levels independent of the vehicle. Modules with a negative $Z_{summary}$ value were considered as on-modules, which might reflect the pharmacologic actions of the three drugs. Compared with the vehicle, 12, 22, and 8 on-modules were detected in the BA, JA, and UA groups, respectively. A complete listing of these on-modules is available in **Supplementary Table S3**. On the other hand, 11, 20, and 7 off-modules with a positive $Z_{summary}$ value were detected in the BA, JA, and UA groups, respectively.

Significant biological functions of on-modules

The GO function and KEGG pathway enrichment analysis revealed a wide range of biological functions associated with the on-modules in the three drug groups. All of the significantly enriched GO terms and pathways ($P < 0.05$) of the on-modules are provided in **Supplementary Table S3**. To specify and simplify the biological functions of the three drugs, we classified the GO terms and pathways based on the GO slim³⁵ and KEGG functional hierarchies (**Figure 4**). For the on-modules in the BA group, the top three GO function categories were metabolism (16.8%), development (9.3%), and cell communication (7.5%); and the top three pathway categories were signal transduction (29%), cancer-specific types (29%), and endocrine system (12%; **Figure 4a,b**). As for the on-modules in the JA group, the top three GO function categories were metabolism (18.9%), binding (8.5%), and death (5.4%); and the top three

pathway categories were cancer-specific types (17%), replication and repair (14%), and signal transduction (14%; **Figure 4c,d**). As for the on-modules in the UA group, the top three GO function categories were metabolism (13.2%), development (9.6%), and binding (9.2%); and the top three pathway categories were cancer-specific types (33.3%), signal transduction (15.2%), and cellular community (12.1%; **Figure 4e,f**). Among the top 10 GO categories, catalytic activity, hydrolase activity, and phosphoprotein phosphatase activity were unique in the BA group, death and cell death were unique in the JA group, and cell differentiation was unique in the UA group.

Moreover, we also compared the overlapping GO terms and pathways of the on-modules in the three drug groups (**Figure 4g,h**). BA and JA shared 19% GO terms and 24% pathways, BA and UA shared 18% GO terms and 32% pathways, whereas JA and UA shared 31% GO terms and 44% pathways. This indicated that JA and UA had more overlapping GO terms and pathways.

UAMs in the three groups

Furthermore, six modules of BA (i.e., the BA_8, BA_11, BA_12, BA_15, BA_20, and BA_22 modules), nine modules of JA (i.e., the JA_5, JA_6, JA_9, JA_13, JA_25, JA_28, JA_30, JA_32, and JA_41 modules), and two modules of UA (i.e., the UA_13 and UA_14 modules) seemed to be unique compared with both vehicle and the other two drug groups, which might differentiate the mechanism of action of the three drugs. Among the genes in these UAMs, DUSP4, FZD7, POU2F1, and MET in BA_8, GPX2 and

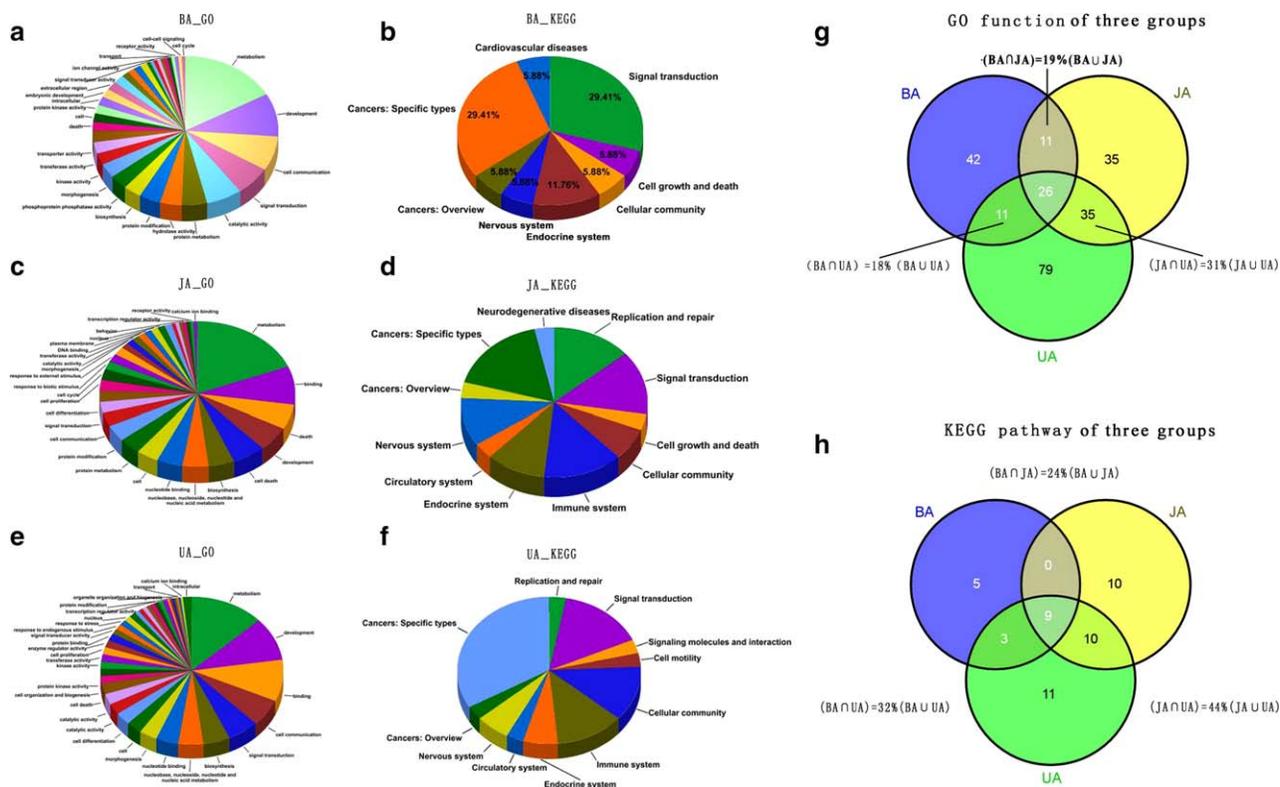


Figure 4 Classification of significant biological functions for the three drug groups are shown. (a–f) The classification of significantly enriched Gene Ontology (GO) terms and pathways ($P < 0.05$) for the baicalin (BA) (a & b), the jasminoidin (JA) (c and d), and the ursodeoxycholic acid (UA) (e and f) groups. (g) The overlapped circles show the significantly enriched GO terms for the three groups. (h) The overlapped circles show the significantly enriched KEGG pathways for the three groups.

JUND in BA₁₁, LDB1 and vascular endothelial growth factor-A in BA₁₂, HTR2C in BA₂₀, CASP7 in JA₅, GPX2 and BAD in JA₆, RARA and E2F1 in JA₂₅, NKD1 in JA₂₈, and DUSP10 in UA₁₃ were significantly differentially expressed compared to vehicle-based on the one-way analysis of variance. The UAMs of each group are listed and visualized according to the module color in **Figure 5**.

Divergent biological functions of the three drugs

To characterize the variant biological functions of the three drugs, we compared the GO functions and pathways of the UAMs in the three groups. The top five significantly enriched GO terms and pathways of the UAMs are listed in **Figure 5**. There were no overlapping GO terms among the three groups; two pathways were shared by BA and JA (i.e., MAPK signaling pathway and colorectal cancer pathway). Based on GO slim classification, BA had more effects on cell communication (4 terms) and signal transduction (3 terms); JA exerted more impacts on cell proliferation (3 terms), nucleobase, nucleoside, nucleotide, and nucleic acid metabolism (3 terms), and binding (3 terms); whereas UA might act more on cell organization and biogenesis (2 terms; **Figure 5**). With respect to pathways, 3, 14, and 0 pathways were enriched by the UAMs in BA, JA, and UA groups, respectively. Except for two overlapping pathways, BA acted on Hedgehog signaling pathway, and JA impacted progesterone-mediated oocyte maturation, melanoma,

prostate cancer, mismatch repair, etc. (**Figure 6a**). Therefore, these three drugs had divergent pharmacologic actions in treating cerebral ischemia.

Western blotting validation

MAP2K6 is an MAPK, which is involved in many pathological processes, such as cerebral ischemia.³⁷ In this study, MAP2K6 was clustered into modules of all the three drug groups. Western blotting analysis showed that the expression level of MAP2K6 increased significantly in all the three groups compared with the vehicle (**Figure 6b**).

DISCUSSION

Modularity has been deemed as a fundamental concept of disease and drug-target networks.³⁸ Studies with a modular design may help to deconstruct complex networks and reveal the relationships between drug actions and disease outcomes.⁵ In this study, a low MCR was obtained among the three drug groups, indicating a difference in their pharmacologic actions globally. From the modular perspective, variant drug-induced coexpression patterns were also noted. BA, JA, and UA modules were all associated with extensive biological functions, including GO functional categories of metabolism, development, and binding, as well as pathway categories of signal transduction, cancer-specific types, etc. These module-enriched functions may provide

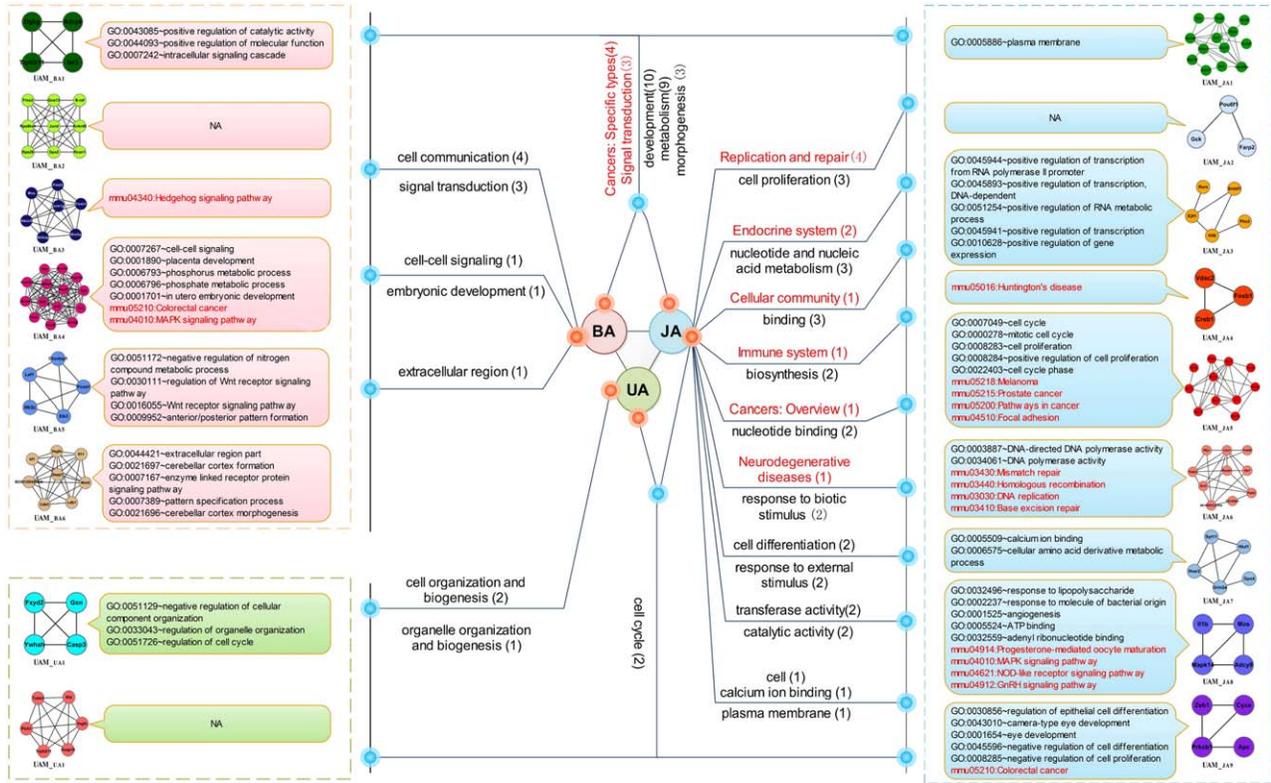


Figure 5 The unique allosteric modules (UAMs) and their significant biological functions. Modules in the orange dotted box are identified from the baicalin (BA) group, the modules in the light blue dotted box are identified from the jasminoidin (JA) group, and the modules in the light green dotted box are identified from the ursodeoxycholic acid (UA) group. The top five significantly enriched functions (black font color represents the Gene Ontology (GO) terms and the red font color represents the KEGG pathways) of each module are listed. These common and divergent biological functions of each group are classified and visualized in the middle of this figure (the black font color represents GO terms' classification and the red font color represents KEGG pathways' classification; the number of terms in a certain category are also listed). NA, not applicable.

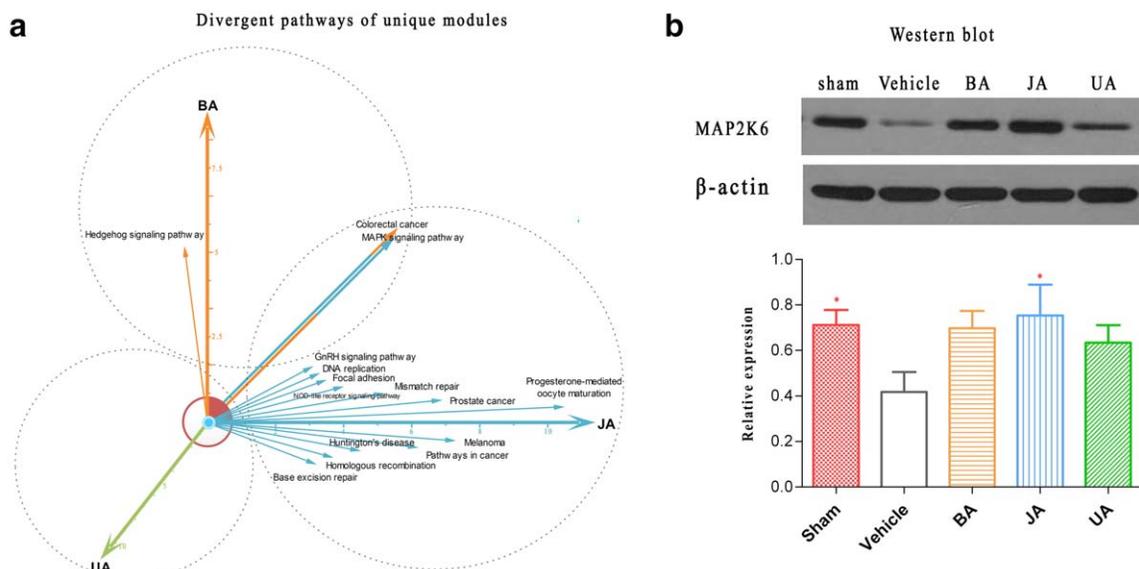


Figure 6 (a) Schematic diagram of the contributing pathways of the three drug groups. The top five enriched pathways of the unique allosteric modules (UAMs) are listed. The orange color lines represent pathways enriched by the UAMs in the baicalin (BA) group, and the light blue lines represent the UAMs in the jasminoidin (JA) group; no pathway is enriched by the UAMs in the ursodeoxycholic acid (UA) group. The length of these lines indicates its approximate enriched $-\log(P)$ value. (b) Western blotting analysis indicates the active patterns of mitogen-activated protein kinase 6 (MAP2K6) under different conditions. * $P < 0.05$ vs. vehicle. MAPK, mitogen-activated protein kinase.

useful implications on the overall difference in the pharmacologic effects of the three drugs at a systems level.

Both on-modules of three drugs enriched extensive functions and pathways, involving their known mechanisms, such as the MAPK pathway for BA and JA,^{16,23} and anti-apoptosis for BA and UA.^{17,25} Not surprisingly, there were both common and unique functions of different drugs on the same disease. In order to analyze the pharmacologic mechanisms in depth and in detail, the CAMs and on-modules should be considered. In this study, two modules (JA_22 and JA_18) were conserved in the vehicle group, which were not affected by these drugs. The activated modules with different drug-induced coexpression patterns may reflect disease-related pharmacologic mechanisms. Similarly, the activated modules may also be conserved in different drug groups; for example, BA_5 module was conserved in both the JA and UA groups, and this module significantly enriched the transcription activator activity, MAPK signaling pathway, and neurotrophin signaling pathway, which have been shown to be closely related to cerebral ischemia.³⁹ These commonly presented modules may be universal therapeutic targets of the three drugs in the treatment of cerebral ischemia.

UAMs can be found by contrastive analysis among different groups based on the drug-induced specific coexpression patterns, which may discriminate the precise details about the actions of different drugs. No overlapping enriched GO terms were found among the UAMs of the BA, JA, and UA groups, demonstrating the unique characteristics of these modules. Except for some basic regulations of molecular functions and cell biological processes, cerebral ischemia-related functions were enriched by these UAMs; for example, BA enriched phosphate metabolic process and Wnt receptor signaling pathway,⁴⁰ whereas JA enriched angiogenesis⁴¹ and calcium ion binding.³⁹ In terms of pathways, MAPK signaling pathway and colorectal cancer were enriched by the UAMs of both the BA and JA groups. Besides, BA also acted on Hedgehog signaling pathway, and JA exerted an impact on Huntington disease, melanoma, mismatch repair, etc. Thus, these unique modules may be used to differentiate the distinct actions of different drugs in treating the same disease.

Significant differential expressed genes in the UAMs of BA, JA, and UA were found to be important for cerebral ischemia therapy. Vascular endothelial growth factor-A in BA_12 were shown to be a target for regulates angiogenesis after ischemic stroke.⁴² BAD in JA_6 was known to play an important role in Bad and Bcl-X(L) interaction-affected neuroprotection.^{43,44} E2F1 in JA_25 plays an important role in modulating neuronal death in response to excitotoxicity and cerebral ischemia.⁴⁵ These important genes in UAMs may provide new clues in cerebral ischemia therapy.

To identify the similar or disparate functions of different drugs, gene expression profiles have been widely used when comparing drug responses, but most previous studies merely focused on the expression difference or chemical structure similarity of a single gene.^{46–48} However, it has been demonstrated that a complex disease is rarely caused by a single gene, but a cluster of functionally related genes.⁴⁹ Modules are considered to be stable groups in

biological networks, and the module biomarkers may be robust, which are not likely to be affected by individual gene expression changes.⁵⁰ Thus, a coexpressed module may provide more implications to infer drug actions.⁵¹ The CAMs and UAMs of different drugs may serve as universal or specific targets in disease treatment. Based on these responsive modules, we may identify both similar and diverse actions of different drugs in treating the same disease, which cannot be easily obtained by analyzing a single gene.

Our module-based analysis may provide a framework to compare the actions of multiple drugs in treating the same disease, but some limitations also exist. For example, quantitative analysis was absent in modular function comparisons that were mainly based on functional annotations, which might restrict the precise assessment of similarities between drugs. In addition, the dynamic variations of modules among different groups were not evaluated, which should be taken into account in future studies.

In conclusion, both CAMs and UAMs of BA, JA, and UA were identified in mice anti-ischemic networks, which may serve as universal and specific therapeutic targets of the three drugs. It is demonstrated that the modules of each drug are related with several divergent biological functions. Our module-centric analysis may provide unique insights into the comparison of pharmacologic mechanisms associated with multiple drugs.

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